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Validation of forced degradation and stability-indicating studies of a nanoformulation using Spectroscopic technique

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Abstract: Analytical method validation plays a vital role in pharmaceutical development by ensuring the accuracy, precision, and reliability of results obtained for drug substances and nanoformulations. This study presents the development and validation of a simple, sensitive, and robust UV spectrophotometric method for the quantitative estimation of drugs in nanoformulations in accordance with International Council for Harmonisation guidelines. The method was systematically evaluated for specificity, linearity, accuracy, precision, range, robustness, and limits of detection and quantitation to confirm its suitability for routine application. Forced degradation studies were performed under hydrolytic, oxidative, photolytic, and thermal stress conditions to assess the stability-indicating capability of the method and to elucidate potential degradation pathways. Additionally, stability studies were conducted to investigate the effects of storage conditions, pH variations, dilution, and temperature on the stability of the nanoformulation. Statistical analysis supported the consistency and reproducibility of the experimental data. The validated method demonstrated reliable performance for bulk drug and nanoformulation analysis. Overall, the proposed approach provides an efficient and cost-effective strategy for analytical method validation and can be successfully applied in routine quality control to ensure the quality, stability, and safety of pharmaceutical nanoformulations.

Introduction

The assessment of the procedure's validity typically involves evaluating its specificity, linearity range, accuracy, precision, and robustness [1]. In the context of particle analysis using laser light diffraction, it's important to note that specification is not applicable because differentiated from dispersed particles without the use of microscopic techniques. Instead of exploring a linear relationship between concentration and response or employing a mathematical model for interpolation, this procedure requires defining a concentration range where the measurement results remain relatively stable. Concentrations below this range may introduce errors due to a poor signal-to-noise ratio, while concentrations above it may lead to errors caused by multiple scattering. The specific range primarily depends on the instrument's hardware, and accuracy should be confirmed through proper instrument qualification and comparison with microscopy. Precision can be assessed by determining repeatability. The achievable repeatability of the method largely depends on the material's characteristics, and the required repeatability varies depending on the measurement's purpose. So, it is not feasible to specify a universal limit in this chapter, as repeatability can vary significantly from one substance to another. However, it is considered good practice to aim for acceptance criteria for

repeatability, such as a percentage relative standard deviation (RSD) of ≥10.0% for any central value of the distribution [1]. For values below 10 μm, this criterion should be doubled. Robustness can be tested during the selection and optimization of the dispersion media and forces, and changes in particle size distribution can be used to monitor alterations in dispersing energy [2].

Forced degradation or stress testing can be used to establish specificity when developing stability-indicating techniques, especially if less amount of information about possible degradant products is accessible. These works disclose the processes of degradation and the degradation products that may develop during storage. Forced degradation study supports pharmaceutical formulations in areas including dosage forms development, production, and packaging, in which chemical behaviour can be leveraged to enhance a therapeutic product. The stability test, known as forced degradation, is used to demonstrate the significance of a stability-indicating analytical method developed with high-performance liquid chromatography (HPLC), that is, a single analytic method capable of distinguishing degradant peaks from drug substance/drug product peaks [3, 4]. Three types of stability studies must be undertaken for the purpose to assess the storage time of a formulation, which are accelerated stability, intermediate stability, and regulated room temperature stability. There are numerous alternatives available, including a 6-month expedited study, another 12- to 24-month intermediate stability study, and a 12- to 24-month controlled room temperature stability study. The active pharmaceutical ingredient or drug formulation will degrade and develop new compounds, known as impurities, throughout the stability study. This is used to determine the inherent stability of a molecule [5].

The concept of validation was first proposed by two Food and Drug Administration (FDA) officials, Ted Byers and Bud Loftus, in 1979 in the USA, to improve the quality of pharmaceuticals. It is proposed in direct response to several problems in the sterility of the large volume parenteral market. The first validation activities were focused on the processes involved in making these products, but quickly spread to associated processes including environmental control, media fill, equipment sanitization, and purified water production. The concept of validation is first developed for equipment and processes and derived from the engineering practices used in the delivery of large pieces of equipment that would be manufactured, tested, delivered and accepted according to a contract. The use of validation spread to other areas of industry after several large-scale problems highlighted the potential risks in the design of products. The most notable is the Therac-25 incident. Here, the software for a large radiotherapy device is poorly designed and tested. In use, several interconnected problems led to several devices giving doses of radiation several thousands of times higher than intended, which resulted in the death of three patients and several more being permanently injured [6]. To develop simple, precise, robust, sensitive, and accurate UV-Spectrophotometric method for the estimation of nanoformulation. The appropriate validation of analytical methods has become an essential part of successful drug development and characterization. Validation of a method involves using experimental design to prove that the method can produce accurate and precise results within the scope of its intended use. Understanding the application and limitations of the test method will allow for accurate assessment of sample information, ranging from process outputs to commercial release testing and many steps in between. If the method validation has not been performed or has been performed in an inadequate manner, the method is not proven to provide reliable data. The key aspect of method validation is the determination of the scope of the method being validated and how that scope will dictate what is to be assessed during validation. This challenge presents itself in the manufacturing environment [7].

Validation: Is performed to demonstrate that the procedure is suitable for its intended use. It shows that the generated result is reliable and accurate. Validation, in its relatively brief history spanning around 50 years, entails the meticulous establishment of documented proof that a process or system possesses the capability to consistently and reliably generate a product with nearly identical characteristics [8]. This validation concept has demonstrated its immense value across various stages of the manufacturing process, contributing to product preservation, enhancement, and the adoption of innovative techniques [9]. As the use of new products

that yield results akin to their predecessors gains prominence, validation has emerged as a pivotal research focus. In current times, validation finds widespread application across diverse industries, including research, medicine, and technology development (involving software, computers, and data collection tools). The validation process is intrinsically linked to technological advancements. It encompasses the systematic collection and evaluation of data throughout different process stages, thereby establishing scientific evidence attesting to a process's consistent capacity to deliver quality on par with the findings of the correlation study. Employing validated methods is pivotal for researchers aiming to demonstrate the qualifications and competencies of new products. This encompasses various forms of validation, such as prospective, concurrent, retroactive, and revalidation, depending on the context. Activities associated with validation studies can be categorized into three distinct phases: The pre-validation phase, the process validation, and the validation maintenance, with statistical process control serving as a viable method of execution. Nonetheless, it's crucial to acknowledge that validation is not without its limitations. One limitation lies in the necessity to identify a common element between the product undergoing validation and the reference product. In certain domains, like Geodesy, validation must adhere to the same zero point to maintain accuracy and consistency [9, 10].

Validation parameters

Specificity or selectivity: Specificity is the ability to assess analyte and equivocally in the presence of expected components (impurities), which can be degradant impurities or Matrix. Selectivity is checked by examining chromatographic blanks (from a sample that is known to contain no analyte) in the expected time window of the analyte peak. And the raw data for selectivity will be recorded in the raw data in approved formats [11].

Linearity: It is the ability to obtain test results that are directly proportional to the concentration of analyte in the sample. Linearity is determined by injecting a series of standards of stock solution/diluted stock solution using the solvent/mobile phase, at a minimum of five concentrations in the range of 50%-150% of the expected working range. The linearity graph will be plotted. Using Microsoft Excel or software on the computer (Concentration vs. Peak Area Response), and which will be attached to the respective study files [12].

Accuracy: Is the closeness of agreement between the value which is accepted (either accepted reference value or conventional true value) and the value found. Accuracy is measured by spiking the sample matrix of interest with a known concentration of analyte standard and analysing the sample using the “method being validated.” The procedure and calculation for accuracy (as % recovery) will be varied from matrix to matrix, and it will be given in the respective study plan or amendment to the study plan. The recovery studies were carried out three times; Chromatogram is recorded and % Recovery and Mean % Recovery is calculated [13].

Precision: Is the closeness of agreement between a series of measurements obtained from multiple samplings of the same sample homogeneous under prescribed conditions. It is considered at three level repeatability, intermediate precision, and reproducibility. Precision is measured by injecting a series of standards or analysing a series of samples from multiple samplings from a homogeneous lot. From the measured standard deviation and mean values [12], precision as % RSD is calculated. The raw data for precision will be recorded in the approved format, and the acceptance criteria for precision will be given in the respective study plan or amendment to the study plan. Precision can also be calculated by using the Horwitz equation. The acceptable % RSD results for precision may be based on the Horwitz equation, an exponential relationship between the among-laboratory relative standard deviation (RSDR) and concentration (C): The Horwitz curve has been empirically derived and has been proven to be more or less independent of analyte, matrix and method of evaluation over the concentration range C= 1 (100%) to C = 10^{-9} by the evaluation of vast numbers of method precision studies. The calibration and validation of analytical methods - A sampling of current approaches modified Horwitz values for repeatability CV given under may be used for guidance. If measured repeatability is outside those values, a suggested explanation must be submitted for consideration. Six replicates of standard solution were prepared and % RSD is calculated [15].

Limit of detection and limit of quantitation: Limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified as an exact value. It is expressed as the concentration of analyte (percentage or parts per billion). LOQ is the lowest amount of sample that can be quantitatively determined with a suitable precision and accuracy. It is a parameter of a quantitative assay for low levels of compounds in a sample and is used for determining impurities (or degradation products). The noise-to-signal ratio for LOQ should be 1: 10. Determination of LOD and Limit of Quantitation (LOQ) from detector linearity experiments (applicable to only instrument sensitivity) [16]. LOD and LOQ values are calculated manually by taking Noise to signal ratio of the lowest/ known concentration of linearity samples, and it will be expressed in µg/ml or ppm. To calculate in %, values of LOD and LOQ will be multiplied by 100/lowest or known concentration of test item (mg/L) taken for analysis of that particular, i.e., or impurity analysis [17].

Stability: Several analytes readily decompose prior to chromatography investigations, for example, during the preparation of the sample solutions, during extraction, clean-up, phase transfer, and during storage of prepared vials. Under these circumstances, method development should investigate the stability of the analyte. The accuracy test takes care of stability. It is required to mention in the method how long a sample, after extraction can be stored before final analysis, based on the duration taken for the accuracy test [18].

Range: The range of an analytical method is the interval between the upper and lower levels that have been demonstrated to be determined with precision, accuracy and linearity using the set method. This range will be the concentration range in which the Linearity test is done [19].

Robustness: Measurement of the capacity to remain unaffected by small but deliberate variations in method parameters and proper indication of its reliability during normal usage. It is demonstrated by making small deliberate changes to the operating parameter [20].

Forced degradation studies of drug and their nanoformulations: Forced degradation studies, or stress testing, are required by regulations and scientific necessity during the initial method development process, according to ICH Q1A (R2) and ICH Q2 (R1). Although regulations mandate that these studies must be performed, the guidance does not provide directions on procedures or conditions to use. The actual protocols employed appear to vary widely in different organizations. An overview of the chemistry fundamentals of drug forced degradation studies and best practices can be found in books, articles, and other resources [21]. Forced degradation studies are performed to investigate the major degradative pathways of the DS and DP and support the initial development of the DS stability indicating methods. High stress conditions are employed to subject DS and DP to conditions more severe than accelerated. Forced degradation studies are conducted under high temperature, humidity, acid/base, oxidative, and light conditions in solid and solution states. **Table I** lists an example set of stress conditions for DS. An important goal of forced degradation studies is to generate a potential level of degradation products that may form during manufacturing during stability storage. Typically, forced degradation studies are conducted until a 5.0%-20.0% loss of API is observed. This range is set to produce a reasonable amount of degradation products to facilitate method development and to avoid secondary degradation products from an unstable degradant (which are not observed under actual stability conditions). The conditions should be selected based on the physicochemical properties of the individual DS and DP [22].

International Conference on Harmonization (ICH) guidelines for forced degradation studies: The ICH guideline indicates that stress testing is designed to determine the intrinsic stability of the molecule by establishing a degradation pathway in order to identify the likely degradation products and to validate the stability-indicating power of the analytical procedure used (**Table 2**). The ICH guidelines ‘stability testing of new drug substances and products requires that stress testing be carried out to elucidate the substance [23]. It suggests that the degradation products that are formed under a variety of conditions should include the effect of temperature, humidity, where appropriate oxidation, photolysis, and susceptibility to hydrolysis across a

wide range of pH values [24]. The ICH guidelines entitled 'Impurities in New Drug Products emphasizes on providing documented evidence that analytical procedures are validated and suitable for the detection and quantification of the degradation product. It is required that the analytical method be validated to demonstrate that impurities unique to the new drug substance do not interfere with or are separated from specified and unspecified degradation products in the drug products [25]. The purpose of forced degradation testing studies is to evaluate the overall stability of the material for method development purposes and/or degradation pathway elucidation. This testing may involve the drug substance alone and/or in simple solutions/suspensions to validate the analytical procedures. Under forcing conditions, decomposition products may be observed that are unlikely to be formed under the conditions used for confirmatory studies. This information may be useful in developing and validating suitable analytical methods. If, in practice, it has been demonstrated that they are not formed in the confirmatory studies, these degradation products need not be further examined [26].

Table 1: An example of stress conditions for nanoformulation of drugs

Factor being studied	Conditions	Time
Light	i. Open dish ii. In the immediate container iii. In the secondary container	Q1B exposure levels (2x to 10x)
Heat (solution)	60°C	Up to 24 hr.
Heat (solid)	50°C, 60°C, 70°C, 80°C	Up to 24 hr.
Accelerated stability	i. Open dish 40°C/75% RH or greater ii. In containers immediate container	Up to 7 days
Peroxide	i. 3% peroxide at RT* ii. 3% peroxide at <40°C	i. 30 mins to 2 hr. ii. 30 mins to 2 days
Acid (solution)	i. 0.1 - 1 N HCl (at RT) ii. 0.1 - 1 N HCl (at 60°C)	i. 30 mins to 2 hr. ii. 30 mins to 2 days
Base (solution)	i. 0.1 - 1 N NaOH (at RT) ii. 0.1 - 1 N NaOH (at 60°C)	iii. 30 mins to 2 hr. iv. 30 mins to 2 days

Table 2: International Conference on Harmonization guidelines for forced degradation studies

Guidelines	Title
Q1A (R2)	Stability testing of new drug substance
Q1B	Photostability study of new drug substance
Q2 (R1)	Validation of analytical procedure
Q3A(R2)	Impurities new drug substance
Q3B(R2)	Impurities new drug products
M4Q(R1)	The common technical document for the registration of pharmaceuticals for human use

Reasons for carrying out forced degradation experiments: As shown in **Figure 1**, and [3, 10], are to:

1. develop and validate stability-indicating methodology for drug components and drug products.
2. find out the degradation pathways of the drug component and the drug products.
3. differentiate degradation products those are related to drug products from those that are generated from excipients in a formulation.
4. search for the mechanisms of degradation such as hydrolysis, oxidation, thermolysis, or photolysis of the drug component and drug product.
5. generate a degradation profile that would be similar to the one observed in a formal stability study under ICH conditions.
6. determine whether a drug component or a drug product is intrinsically stable.
7. study the chemical properties of the drug component or the drug product.
8. produce stable formulations, and to
9. determine the structure of degraded products and to solve stability-related problems

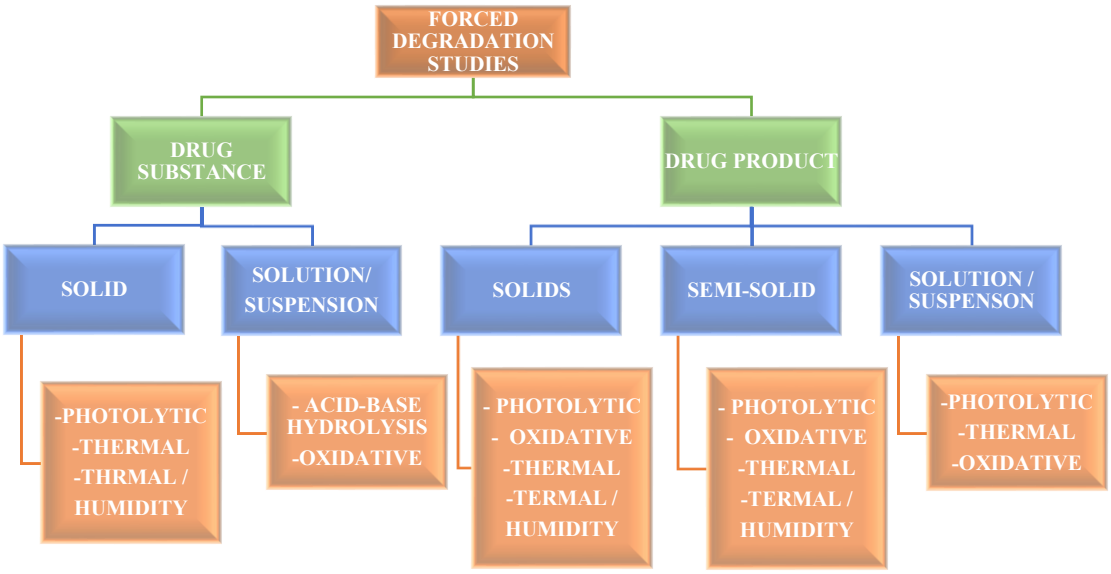

Figure 1: Common conditions used in conducting forced degradation study [10]

Types of studies carried out in forced degradation

Hydrolysis degradation: Hydrolytic conditions are prevalent in various pH ranges and are responsible for the degradation of many substances. Hydrolysis is a process in which a substance, such as a pharmaceutical drug, reacts with water, resulting in the formation of breakdown products with different chemical compositions. Water, whether acting as a solvent or present as moisture in the atmosphere, plays a significant role in causing the degradation of numerous drugs, for instance, aspirin undergoes hydrolysis when it comes into contact with water, leading to the formation of salicylic acid and acetic ting should not exceed a duration of more than seven days. Following degradation, the sample is neutralized using an appropriate acid, base, or buffer before injection to prevent further decomposition [27].

Oxidation degradation: Oxidative conditions for forced degradation typically involve the use of hydrogen peroxide as a widely employed oxidizing agent. In addition to hydrogen peroxide, metal ions, oxygen, and radical initiators such as azobiisobutyronitrile (AIBN) can be utilized. The selection of the oxidizing agent, its concentration, and the specific conditions employed depend on the drug's molecular structure, allowing for tailored oxidative testing. It's worth noting that many drug substances are prone to auto-oxidation [28], a process where they undergo oxidation even under normal storage conditions, primarily due to exposure to ground state elemental oxygen. Auto-oxidation is characterized by a free radical reaction that necessitates the presence of a free radical initiator to initiate the chain reaction. Initiators for drug substances can include hydrogen peroxide, metal ions, and trace levels of impurities. The choice of initiator, its concentration, and the testing conditions are determined by the unique characteristics of the drug substance. For example, drug solutions are typically subjected to oxidizing conditions involving 0.1% to 3.0% hydrogen peroxide at a neutral pH and room temperature for a duration of seven days. In some cases, testing may extend until a maximum of 20.0% degradation is achieved, allowing for the potential generation of relevant degradation products [29].

Photolytic degradation: Photolytic conditions are crucial to assess the photo stability of drug substances, ensuring that exposure to light does not lead to undesirable changes. Photo stability studies are conducted to intentionally generate primary degradation products of a drug substance by subjecting it to UV or fluorescent light conditions. These conditions are often recommended by the International Council for Harmonisation

(ICH) guidelines. To perform photostability testing, drug substance samples and solid/liquid drug products should be exposed to a minimum of 1.2 million lux-hours and 200 watt-hours per square meter (Wh/m^2) of light. The light used typically falls within the wavelength range of 300 to 800 nanometres (nm), which is known to induce photolytic degradation. The maximum recommended illumination duration is 6 million lux-hours [30]. Under these light stress conditions, photodegradation can occur through a free radical mechanism, affecting functional groups within the drug molecule. Functional groups such as carbonyls, nitroaromatics, nitrates, alkenes, aryl chlorides, weak C-H bonds, O-H bonds, sulphides, and polyenes are known to introduce drug photosensitivity and are susceptible to photolytic degradation [31].

Thermal degradation: Thermal degradation studies, encompassing both dry heat and wet heat conditions, necessitate more rigorous testing conditions compared to the accelerated testing conditions recommended by ICH Q1A guidelines. These studies involve exposing solid-state drug substances and drug products to dry or wet heat, while liquid drug products are typically subjected to dry heat. In some cases, experiments may be conducted at higher temperatures for shorter durations to assess the impact of temperature on thermal degradation. The behaviour of a substance undergoing thermal degradation concerning temperature can be analysed using the Arrhenius equation [32]. Thermal degradation studies are typically carried out within a temperature range of 40-80°C. These studies aim to provide insights into how temperature influences the degradation process, helping to assess the stability of drug substances and products under different thermal conditions [3].

Table 3: Conditions commonly employed for forced degradation

Degradation type	Experimental condition	Storage conditions	Sampling time (days)
Hydrolysis	Control API (no acid or base)	40°C, 60°C	1, 3, 5
	0.1 M HCl	40°C, 60°C	1, 3, 5
	0.1 M NaOH	40°C, 60°C	1, 3, 5
	Acid control	40°C, 60°C	1, 3, 5
	Base control	40°C, 60°C	1, 3, 5
	pH: 2, 4, 6, 8	40°C, 60°C	1, 3, 5
Oxidation	3.0% H_2O_2	25°C, 60°C	1, 3, 5
	Peroxide control	25°C, 60°C	1, 3, 5
	Azobisisobutyronitrile (AIBN)	40°C, 60°C	1, 3, 5
	AIBN control	40°C, 60°C	1, 3, 5
Photolytic	Light 1 x ICH	NA	1, 3, 5
	Light 3 x ICH	NA	1, 3, 5
	Light control	NA	1, 3, 5
Thermal	Heat chamber	60°C	1, 3, 5
	Heat chamber	60°C/75% RH	1, 3, 5
	Heat chamber	80°C	1, 3, 5
	Heat chamber	80°C/75% RH	1, 3, 5
	Heat control	Room temp	1, 3, 5

Stability study: Stability studies for clinical trial materials (CTMs) are conducted to monitor their CQAs and help identify which formulation will result in a successful candidate for regulatory submission. In the USA, an expiration date is not required on the label of a CTM, but this is required in many countries, such as those in Europe. Forced degradation studies are used to challenge the stability-indicating power of the analytical method [33, 34]. The accelerated studies, which require storage under higher temperatures and humidity compared to normal room-temperature conditions, allow the samples to degrade at a faster rate. Data from accelerated studies may be extrapolated to project the results of a longer-term, controlled-room-temperature study. Most pharmaceutical products exhibit a linear degradation trend. Based on the stability data, a retest period is assigned to the DS, and an expiration date to the DP [35]

Harmonization of regulations quality guidelines for stability studies: Given that stability testing is expensive and labour - intensive, it is imperative to understand the regulatory requirements and their intent to avoid

unnecessary studies. In addition, regulations vary significantly from country to country, which impacts the cost of new drug development and the registration timeline for a global product launch. So, in the early 1990s, the ICH of Technical Requirements for Pharmaceuticals for Human Use was established consisting of representatives from regulatory agencies and scientists from the pharmaceutical industries, is formed. The council's role is to harmonize the quality requirements for pharmaceuticals to minimize redundant testing and reduce the time and cost of new product development. The ICH stability guideline, ICH Q1, is the first quality guideline established by the council, indicating the significant need for harmonization of global stability requirements [36]. The ICH initially harmonized three geographic regions: The United States, the European Union, and Japan, which encompassed Zone 1 and Zone 2 of the World Health Organization (WHO) climatic zones. However, many countries outside Zone 1 and 2 joined the ICH, or voluntarily adopted these guidelines later with various modifications. The current stability guideline (Q1A [R2]) harmonized the storage conditions, frequency of required testing, and the minimum amount of data needed for registration (6-8). **Table 2** lists the stability storage conditions established by ICH Q1A (R2) [27].

Different stability studies

Storage stability: The selected formula was tested at various time intervals for homogeneity, particle size, and polydispersity index. This test is conducted at ambient temperature to examine selected nanoformulation's physical stability and storage behaviour [37]. It is intended to demonstrate the kinetic stability of formulations system. There were no signs of sedimentation or liquid separation, and the nano micelle formulations remained clear for three months when observed by visualization. There is also no significant rise in particle size and PDI after three months. The nanoformulation dispersion system should be highly stable, with outstanding clarity and no evidence of precipitation. The stability of nanoformulations decreases as the temperature approaches 40ºC. After 24 hours, a turbid formulation has formed because the high temperature negatively impacts the stability of nanoformulations. At high temperatures of more than 40°C, formulations are susceptible to distortion and disintegration, resulting in turbidity in this colloidal dispersion because, as the temperature increases, the CMC increases, then the thermodynamic and kinetic stability decrease [38].

Table 4: Storage conditions established by the ICH Q1A (R2) guidelines for zone

Label Conditions	Storage condition	Storage conditions to be studied
Controlled room temperature	Long term storage	25.0 ± 2°C and 60.0 ± 5% RH
	Intermediate condition	30.0 ± 2°C and 65.0 ± 5% RH
	Accelerated condition	40.0 ± 2°C and 75.0 ± 5% RH
Refrigerated	Refrigerated condition	-3.0 ± 5.0°C
Freezer	Freezing condition	-20.0 ± 5.0 °C

pH stability study: This study is made for a selected formula by adding 0.5 mL to 30 mL of each 0.1 N HCl (pH 1.2) and phosphate buffer (pH 7.4) to achieve a 60-fold dilution. Then monitored by visual investigation, the occurrence of Tyndall phenomena using red laser light and DLS for particle size and PDI analysis at 0, 2, 4, 8, and 24 hr. at 37°C. The experiment worked out in triplicate [39]. This test is created to study the behaviour of chosen nanoformulations when passing through different pH media to simulate varying physiological Ph in the GIT. The formulations should resist degradation and not release the loaded medicine when passing through different physiological pH levels to deliver the drug safely to the systemic circulation. Nano-formulations that are stable in an acidic stomach pH and stable in the intestinal pH, indicating that formulations are resistant to various pH changes and produce stable formulations capable of safely transporting medicines to the circulation. After 2 hrs. of incubation at 37°C, particle size and PDI of nano formulations increased [40]. If it remains within the standard limit for good oral absorption. There is no sign of sedimentation after 8 hrs. when observed by visualization. Therefore, it is stable in human gastric and intestinal conditions [41].

Dilution stability: The dilution test is proposed to determine if a significant amount of the external (continuous) phase could be introduced to the chosen nano-micelle formula without generating stability issues. This test is made by adding 0.5 mL of the selected formula to 3.0 mL (1:6) and 6.0 mL (1: 12) of distilled water to achieve a level of dilution where the concentration of the formulation is equal to 1.0 mg/mL and 0.5 mg/mL, respectively. This addition is made directly after the formula preparation. After resting for one hour, these formulas were examined visually and measured particle size and PDI; the experiment was done in triplicate [42].

The purpose of the dilution test is to determine how the particle size and distribution of certain nanoformulations changed as a result of dilution. This test is to see how the stability of the nano formulations is affected by volume expansion. The results explain that no significant effect on the particle size and PDI of nano formulations were diluted to the degree that concentrations reached 2.0 mg/mL and 1.0 mg/mL, respectively. It can give an idea about the resistance of nano formulations against dilution effects. The nanoformulations are stable at concentrations above the CMC, while disintegration and morphological changes in nanoformulations occur at concentrations below the CMC [43].

Statistical analysis: The General Factorial regression on Minitab software (version21) is used to analyse the factorial design responses at a confidence interval of 0.95. The statistical analysis utilizing ANOVA (analysis of variance) at p-value ≤0.05 is applied to analyse the effect of sonication on the spherical diameters and PDI of the prepared formulas [44].

Table 5: Characteristics of stability studies and testing

Stability study characteristics	Stability testing guidelines
Stability studies must be conducted to meet regulatory expectations	i.Drug regulations require expiration dates to be posted on the levels for all the pharmaceutical products ii.Stability data of the DS and DP are submitted to regulatory agencies
Stability studies fall into different types	i.Formal stability studies are used to justify storage conditions and the expiration dating of packaged product ii.Other structure secondary aur those used to support the primary stability data set iii.Stress studies include stabilities studies and studies that support temperature excursions throughout the supply chain.
Studies are conducted under multiple storage	i.Stability studies are conducted at several environmental conditions based on the desert level storage conditions. ii.For product to be stored at room temperature studies are conducted at room temperature and accelerated conditions intermediate conditions can be studied if sample at accelerated condition may not meet room temperature specification. iii.Refrigerated or freezing conditions are used for products that are not chemical stable at room temperature.
Stability indicating analytical procedures mostly followed	i.Attributes that change over time are required to be assessed instability study ii.The chemical physical microbiological and performance characteristic of DP must meet acceptance criteria throughout the shelf life iii.Forced degradation studies are performed to challenge the stability indicating power of the method and to evaluate the major degradable pathways of the API
Stabilities studies are resource intensive	i.Stability testing consuming of significant part of the internal analytical testing and outsourcing resources of pharmaceutical companies

Conclusion: The proposed method is quite simple, reliable, sensitive, efficient, reproducible, co-efficient, and can be successfully applied to the quantification in bulk and in nano- formulations. The validation method confirms that this is a suitable and feasible approach for estimating the drug in nanoformulation. It can be easily introduced for routine quality control to monitor the assay in the bulk and nanoformulations. The results obtained indicated that the use of a mixture design approach is an inexpensive and versatile method that can reduce the number of experiments and can be used to produce as much information as possible in less time. The simultaneous method developed is found to be quick, easy, and effective as validated per ICH guidelines.

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